[Agr. Biol. Chem., Vol. 34, No. 8, p. 1235~1243, 1970]

Isolation of Bitter Peptides from Tryptic Hydrolysate of Casein and their Chemical Structure†

By Teruyoshi MATOBA, Rikimaru HAYASHI and Tadao HATA

Research Institute for Food Science, Kyoto University, Kyoto

Received February 5, 1970

Bitter peptides were isolated from the tryptic hydrolysate of casein. Fractionation and isolation were carried out using *n*-butanol extraction, acidic precipitation at pH 5.4, gel filtration with Sephadex G-25, ion exchange chromatography with Dowex 50 W and paper chromatography. Three kinds of bitter peptides were purified. The primary structures of these peptides were proposed as follows; BP-I, Gly-Pro-Phe-Pro-Val-Ileu; BP-II, Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys; BP-III, Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys. These peptides were very bitter in a 0.1% solution.

L-Tyrosine, L-phenylalanine and their derivatives were also tasted. The importance of the position of bitter amino acids in the peptide in the development and strengthening of its bitter taste is discussed.

A wide variety of bitter peptides have been found in proteolytic hydrolysates of proteins[1] and in dairy products, especially in some cheeses.[2] However, these peptides have not been fully characterized in terms of their chemical structures, except for the bitter peptides isolated by Fujimaki's group.[3,4] In order to understand the relationship of bitter taste to the chemical configuration of peptides, more detailed knowledge about different bitter peptides is needed.

This paper describes the isolation of and structural studies on the bitter peptides present in the tryptic hydrolysate of casein. The importance of L-phenylalanine and L-tyrosine residues in strengthening bitterness is also discussed.

A preliminary note on this work has appeared.[5]

MATERIALS

Trypsin from bovine pancreas (twice crystallized, salt free) was purchased from Sigma Chemical Company. Carboxypeptidase A (three times crystallized) and B were obtained from Worthington Biochemical Company and Mann Research Laboratories, respectively. These were previously treated with diisopropylfluo-rophosphate.[6] Hammersten casein was purchased from E. Merck AG. Darmstadt. Reagents used for Edman degradation were purified according to procedures described by Edman and Begg.[7] Dimethylallylamine was synthesized by the method of Ahlroth.[8] Tyrosine* ethylester (Tyr. OEt) and

† This report was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April, 1969.

Abbreviation: PTH, 3-phenyl-2-thiohydantoin; CPA, carboxypeptidase A; CPB, carboxypeptidase B.

1) See references 1 and 2 in the preliminary note.[5]

2) J. Schomüller, *Adv. Food Res.*, **16**, 231 (1968).

3) M. Fujimaki, M. Yamashita, Y. Okazawa and S. Arai, *Agr. Biol. Chem.*, **32**, 794 (1968).

4) M. Yamashita, S. Arai and M. Fujimaki, *ibid.*, **33**, 321 (1969).

* All amino acids have L-configuration unless otherwise stated.

5) T. Matoba, C. Nagayasu, R. Hayashi and T. Hata, *ibid.*, **33**, 1662 (1969).

6) J. T. Potts Jr, "Method in Enzymology," Vol. XI, ed. by C. H. W. Hirs, Academic Press, New York, 1967, p. 648.

7) P. Edman and G. Begg, *European J. Biochem.*, **1**, 80 (1967).

8) A. Ahlroth, *Acta Chem. Scand.*, **19**, 2420 (1965).

N-acetyltyrosine ethylester (Ac-Tyr. OEt) were purchased from the Institute for Protein Research, Osaka University, Osaka. N-Acetyltyrosine (Ac-Tyr) was kindly supplied from Dr. Narita, Osaka University. Acetylphenylalanine (Ac-Phe), phenylalanine ethylester (Phe. OEt) and acetylphenylalanine ethylester (Ac-Phe. OEt) was synthesized by the usual procedures.

EXPERIMENTAL METHODS

Detection, concentration and estimation of peptides. Chromatographic elution patterns were determined by subjecting aliquots of column effluents (0.2 ml) to reactions with ninhydrin, in the usual manner. Fractions having bitter tastes were pooled. The solvent was removed by evaporation under reduced pressure in a rotary evaporator at 40~50°C. Residues were lyophilized.

Tasting of bitter peptides. Throughout the purification process, 1~2 mg of a freezed dried sample was dissolved in 1 ml of water and tasted. In tasting amino acids and their derivatives, compounds were dissolved in water by warming and were tasted after being neutralized with diluted HCl or NaOH. Tasting was usually performed by a panel of two persons.

Ion-exchange chromatography. Dowex 50 W×4 (200~400 mesh) was immersed in water several times and fine particles were removed by decantation. The resin was purified by washing it with 2 N NaOH and 2 N HCl, and was converted to the pyridine form and equilibrated with 0.2 N pyridine-acetic acid, pH 3.1. The column (2.5×92 cm) was maintained at 40°C and developed by step-wise elution at a flow rate of 110 ml per hour.

Gel-filtration chromatography. Sephadex G-25 (fine grade, Pharmacia, Uppsala) was used. The gel was immersed in water several times and fine particles were removed by decantation. The column (5×78 cm) was equilibrated and eluted with 0.01 M sodium phosphate, pH 7.5. Desalting of the peptides was performed using the same column equilibrated with distilled water.

Paper chromatography and paper electrophoresis. Purity of the peptides was checked by high-voltage paper electrophoresis and paper chromatography. Electrophoresis was carried out on Whatman No. 1 paper at a potential gradient of 70 V/cm for 45~80 min using a hexane-cooled, vertical strip apparatus. Pyridine-acetic acid-water (1 : 10 : 90) buffer (pH 3.6) or pyridine-acetic acid-water (10 : 0.4 : 90) buffer (pH 6.5) were used routinely. Ascending paper chromatography was routinely performed for 14~16 hr at 20°C on Toyoroshi No. 51 A paper using *n*-butanol saturated with water-acetic acid (8 : 1) or pyridine-*n*-butanol-water (1 : 1 : 1) as solvents.

Paper chromatography was also used for final purification of the bitter peptides obtained by ion-exchange chromatography. Toyoroshi No. 51 A paper (20×20 cm) was washed with 3% acetic acid and dried at room temperature prior to use. Peptide solution was applied to a line on a sheet of this paper and developed. After development, the paper was dried at room temperature and guide strips for the location of bands were cut from both sides of the strips and stained with 0.5% ninhydrin in an acetone solution. Peptides from the main strip were eluted with distilled water, then freeze-dried.

Characterization of peptides. Amino acid compositions of the peptides (about 3 mg) were determined with a Yanagimoto LC-5 S automatic amino acid analyzer, after hydrolysis at 110°C for 20 hr with HCl distilled twice at 109°C (2 ml) in a sealed tube under an argon gas phase.

The C-terminal sequence of the peptides was estimated using carboxypeptidase A or B. The peptide, 0.3~0.5 μmoles in 1 ml of 0.2 M N-ethylmorpholine-acetic acid buffer, pH 8.5, was incubated with a carboxypeptidase solution at 25°C for 15~30 min. The ratio of carboxypeptidase to peptide was approximately 1 : 100. The reaction was stopped by adding 0.5 ml of 1 N HCl. Liberated amino acids were determined in the amino acid analyzer.

Qualitative analysis of phosphorus in the peptides was performed using the method of Allen,[9] with a slight modification. Peptides were incubated with 1 ml of amidol reagent, 0.5 ml of 60% perchloric acid and 0.5 ml of 3.3% ammonium molybdate at 30°C for 20 min. The absorbancy at 650 mμ was measured in a Shimadzu QV-50 spectrophotometer.

Edman degradation. The amino acid sequence of peptides was determined using the Edman degradation method as summarized by Doolittle,[10] with slight

9) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).
10) R. F. Doolittle, *ibid.*, **94**, 742 (1965).

modifications. Peptide samples (usually 1～3 mg) were dissolved in 0.2 ml of pyridine-water (3 : 2, v/v) buffer containing 0.4 M dimethylallylamine (adjusted to pH 9.6 with trifluoroacetic acid). After addition of 10 μl phenylisothiocyanate, the tube was flushed with a gentle stream of nitrogen for a few seconds, then stoppered and incubated at 40°C for 1 hr. For bitter peptide fractions, BP-I and BP-II, the reaction mixture was then evaporated to dryness. Volatile reagents and buffer were removed under a high vacuum at an elevated temperature (50～60°C).[11] Residues were washed three times with 1 ml of benzene then freeze-dried. For BP-III, the coupling reaction mixture was washed three times with 1 ml of benzene then freeze-dried. Cleavage was performed in 10 μl of trifluoroacetic acid for 20 min at 40°C. The thiazolinone which formed was extracted by benzene (1.0, 0.8 and 0.6 ml) instead of ethylene dichloride to prevent partial extraction of the remaining peptide fragment together with the thiazolinone. The residual peptide was dried overnight *in vacuo* and used for the next degradation cycle. The thiazolinone in the benzene phase was dried in a gentle stream of nitrogen and converted to the corresponding thiohydantoin by treating it with 0.3 ml of 1 N HCl at 80°C for 10 min. The thiohydantoin derivative was extracted three times with 0.6 ml of ethyl acetate. The extract was dried in a stream of nitrogen and dissolved in an appropriate amount (100～200 μl) of ethyl acetate. The water phase, left after the extractions, was also dried and dissolved in 100 μl of water. The PTH-amino acids contained in the organic phase were routinely identified by thin-layer chromatography of a precoated silica gel G plate with fluorescence indicator (DC-Fertigplatten Kieselgel F_{254}, Merck) in four different solvent systems, *i.e.* solvent E and D of Edman and Sjöquist,[12] solvent III of Brenner *et al.*[13] and solvent V (*n*-butanol-ethylene dichloride-propionic acid; 45 : 25 : 30). PTH-amino acids in the water phase (PTH-arginine and PTH-histidine) were identified by paper electrophoresis in 0.02 M phosphate buffer, pH 6.2. Spots on the chromatograms were located by illumination with ultraviolet light and with the iodine-azide reaction.[14] Quantitative determination of PTH-amino acid was performed on the basis of the absorbancy at 269 mμ of the organic or water phase.

11) W. R. Gray, "Method in Enzymology," Vol. XI, ed. by C. H. W. Hirs, Academic Press, New York, 1967, p. 469.

12) P. Edman and J. Sjöquist, *Acta Chem. Scand.*, **10**, 1507 (1956).

13) M. Brenner, A. Niederwieser and G. Pataki, "Thin-layer Chromatography," ed. by E. Stahl, Academic Press, New York, 1965, p. 427.

RESULT

Isolation of bitter peptides from casein hydrolysate

a) Tryptic digestion of casein. One hundred grams of casein was warmed in 2 liters of distilled water to dissolve it, then cooled to room temperature. The solution was adjusted to pH 7.5 with 1 N NaOH, then 200 mg of trypsin (dissolved in 2 ml of water) was added. A few drops of toluene were also added to prevent bacterial contamination and the mixture was incubated at 25°C, maintaining a pH of 7.5 by occasionally adding 1 N NaOH. The extent of hydrolysis was followed using the method of Hagihara *et al.*[15]

Bitter taste was detected by tasting an

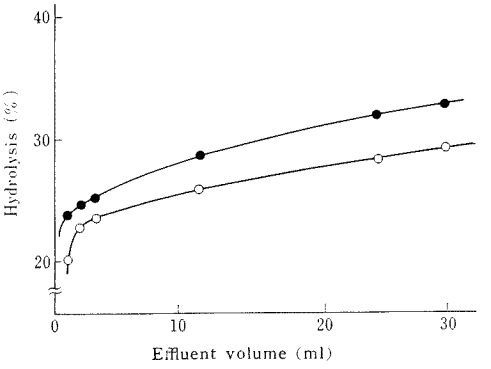

FIG. 1. Time Course for Tryptic Hydrolysis of Casein.

5% Casein was incubated with different amounts of trypsin at pH 7.5 and 25°C. An aliquot of the digestion mixture was removed at various time intervals and the extent of hydrolysis and bitter taste was measured.

●—● The ratio of trypsin to casein; 1 : 125

○—○ The ratio of trypsin to casein; 1 : 250

14) J. Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1953).

15) B. Hagihara, H. Matsubara, M. Nakai and K. Okunuki, *J. Biochem.* (*Tokyo*), **45**, 185 (1958).

aliquot of the mixture. Time courses for proteolytic hydrolysis and development of bitter taste are shown in Fig. 1. Bitter taste appeared with the progress of hydrolysis and reached a maximum after about 20 hr, under the conditions tested.

b) Extract with n-butanol. Immediately after incubation for 20 hr, the hydrolysate was mixed with 2 liters of *n*-butanol, and this mixture was vigorously stirred for 2 hr. The butanol phase was separated from the water phase by centrifugation at 3000 rpm for 10 min, and both phases were collected separately. The water phase was, again, mixed with 2 liters of *n*-butanol and treated with the same procedure as above. Both water and butanol phases were concentrated, then lyophilized. Bitter taste was concentrated in butanol phase.

c) Acid treatment. The dry residue from the butanol extract (14.3 g) was suspended in 500 ml of distilled water and was solubilized by adjusting the pH to 10 with 1 N NaOH under constant stirring. The resulting clear solution was slowly adjusted to pH 5.4 with 1 N HCl under vigorous stirring. The precipitates were removed by centrifugation at 3500 rpm for 10 min and washed by repeating the same procedure. Supernatant solutions were combined, concentrated and lyophilized.

d) Gel-filtration with Sephadex G-25. The freeze-dried residue (8.4 g) was dissolved in 70 ml of 0.01 M sodium phosphate, pH 7.5. Insoluble materials were removed by centrifugation. The supernatant (35 ml) was applied to a Sephadex G-25 column and eluted with 0.01 M sodium phosphate buffer, pH 7.5. The resulting pattern of gel-filtration is shown in Fig. 2. The result showed that the molecular weight of bitter component is under 1000. This gel-filtration chromatography was repeated twice and the bitter fractions were collected, concentrated and lyophilized.

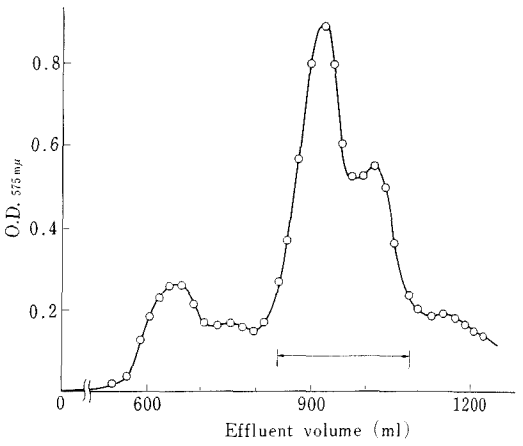

FIG. 2. Chromatography on a Sephadex G-25 Column (5×71 cm).

Bitter fractions are indicated with an arrow.

e) Ion-exchange chromatography with Dowex 50 W. The freeze-dried residue (2.1 g) was dissolved in 30 ml of 0.2 M pyridine-acetic acid buffer, pH 3.1. An aliquot (10 ml) of the solution was applied to a Dowex 50 W×4 column and stepwise developed with the buffers: 0.2 M pyridine-acetic acid, pH 3.1, 1 M pyridine-acetic acid, pH 4.1 and 2 M pyridine-acetic acid, pH 5.0. The chromatogram is shown in Fig. 3. Bitter taste was found in fractions I, III, IV and V. This chromatography was repeated three times and each fraction was separately collected and lyophilized. The yield of fractions I, III, IV and V were 130 mg, 627 mg, 177 mg and 129 mg, respectively. All peptide fractions were subjected to a homogeneity test with paper chromatography. Fraction I revealed a single spot giving a yellow color with ninhydrin, whereas, the other fractions showed one strong and several faint ninhydrin-positive spots (Fig. 4). Fraction I also gave a single spot with paper electrophoresis at pH 3.5 and 6.5. Further purification of fractions III and IV was achieved using paper chromatography.

f) Purification of fractions III and IV with papar chromatography. Freeze-dried materials

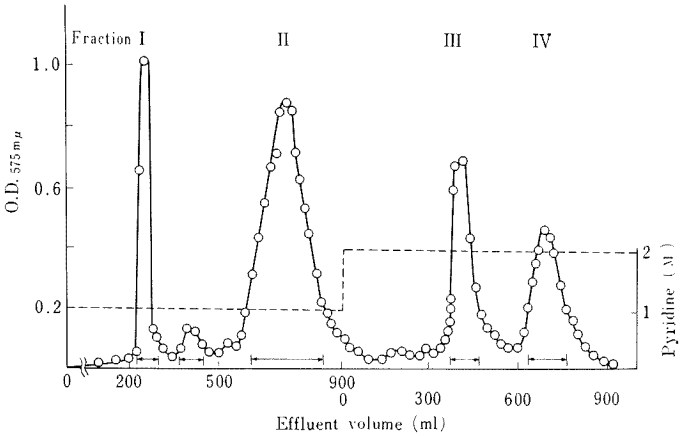

FIG. 3. Chromatography on a Dowex 50 W Column (2.5×92 cm).
Bitter fractions I, III, IV and V were separately pooled as indicated by arrows.

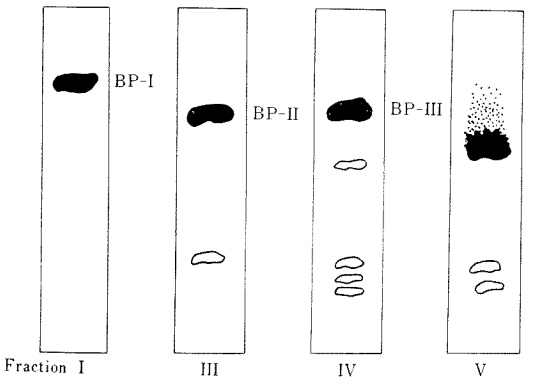

FIG. 4. Paper Chromatography of Bitter Fractions.
Development was carried out using the following solvent system; for BP-I and BP-III, pyridine-*n*-butanol-water (1 : 1 : 1), for BP-II, *n*-butanol saturated with water-acetic acid (8 : 1).

of fractions III and IV were dissolved in a minimum quantity of water and subjected to paper chromatography. Fraction III was developed with *n*-butanol saturated with water-acetic acid (8 : 1). Fraction IV was developed with pyridine-*n*-butanol-water (1 : 1 : 1). From each chromatogram, the main ninhydrin-reactive zone was cut out and its peptides were eluted with water. This paper chromatography was performed repeatedly and each peptide was collected and finally lyophilized. Isolated peptides were homogeneous, as judged by their high voltage paper electrophoreses. Isolation of bitter peptide from fraction V was unsuccessful using paper chromatography.

Isolated peptides were named bitter peptides (BP) I, II and III corresponding to fractions I, III and IV. respectively.

A 0.1% aqueous solution of each peptide was concentrated enough to recognize the bitter taste. Strengths of bitterness were in the order of BP-I, BP-III and BP-II.

Chemical compositions of BP-I, BP-II and BP-III

About 3 mg of the sample peptides were hydrolyzed in 2 ml of HCl, distilled at 109°C. After the removal of HCl under reduced pressure, amino acid compositions were analyzed. Results are given in Table I, where the residues are expressed as molar ratios. BP-I, BP-II and BP-III consisted of 6, 12 and 8 amino acid residues, respectively. The molecular weight, calculated as 628.8 for BP-I, 1384.7 for BP-II, and 980.2 for BP-III, closely approximated the values which were estimated from gel-filtration chromatography.

An abundancy of an amino acid having a

TABLE I. AMINO ACID COMPOSITION OF BITTER PEPTIDES

Amino acid	Residues/Molecules		
	BP-I	BP-II	BP-III
Lys	—	0.987 (1)	1.01 (1)
Asp	0.031	—	0.024
Thr	0.037	—	0.016
Ser	0.049	0.030	0.039
Glu	0.095	1.01 (1)	1.03 (1)
Pro	2.27 (2)	1.94 (2)	1.00 (1)
Gly	1.00 (1)	1.00 (1)	0.030
Ala	0.055	1.02 (1)	0.990 (1)
Val	1.03 (1)	1.81 (2)	0.026
Met	0.011	—	—
Ileu	1.33 (1)	—	0.002
Leu	0.083	—	1.99 (2)
Tyr	—	—	1.02 (1)
Phe	1.10 (1)	3.89 (4)	0.971 (1)
M. W.	628.8	1384.7	980.2

( ) best estimated value.

hydrophobic side chain was a unique characteristic for these peptides. Proline and phenylalanine were contained in common in all the peptides. The absence of tryptophan in the peptides was confirmed by spectrophotometric analysis in the ultraviolet region.

Carr *et al.*[16] denied that phosphorous contributed to the bitter taste of peptides. This was also supported by a phosphorous analysis of about 3 mg of the peptides, which showed the absence of phosphorous.

Sequential analysis of the bitter peptides

The peptides (BP-I, 2.78 μmoles; BP-II, 1.11 μmoles; BP-III, 2.80 μmoles) were subjected to Edman degradation. During consecutive degradations one main PTH-amino acid was released in each cycle. However, the appearance of considerable amount of several PTH-amino acids was observed near the final steps. Degradation was duplicated for each sample. Stepwise results and yields (average of duplicate analyses) are summarized in Table II~IV, where yields are expressed as percentages of the material found in the preceding step. The average yield in each degradation cycle was 66% for BP-I, 102% for BP-II and 80% for BP-III. The relatively low yield for BP-I may be due to partial extraction of the remaining peptide during the extraction procedure of the thiazolinone, because the peptide consisted of only a neutral amino acid having a hydrophobic side chain.

TABLE II. YIELD OF PTH-AMINO ACID AT EACH DEGRADATION STEP OF BP-I

Step	PTH-Amino acid (μmoles)	Yield (%)	Result
1	2.58	92.5	Gly
2	1.58	61.2	Pro
3	0.70	44.3	Phe
4	0.52	74.3	Pro
5	0.39	75.0	Val
6	0.19	48.7	Ileu
7	Trace	—	—
Average yield		66.5	

TABLE III. YIELD OF PTH-AMINO ACID AT EACH DEGRADATION STEP OF BP-II

Step	PTH-Amino acid (μmoles)	Yield (%)	Result
1	1.24	101	Phe
2	1.14	94.0	Phe
3	1.08	98.2	Val
4	0.89	85.8	Ala
5	0.78	82.9	Pro
6	0.79	101	Phe
7	0.28	35.1	Pro
8[a]	1.42	176	Glu
9	2.03	143	Val
10	2.85	140	Phe
11	2.05	71.2	Gly
12	1.89	92.2	Lys
13	Trace	—	—
Average yield		102	

[a] Pooled with 0.55 μmoles from another preparation.

16) J. W. Carr, T. C. Loughheed and B. E. Baker, *J. Sci. Food. Agr.*, **7**, 629 (1956).

TABLE IV. YIELD OF PTH-AMINO ACID AT EACH DEGRADATION STEP OF BP-III

Step	PTH-Amino acid (μmoles)	Yield (%)	Result
1	1.82	65.0	Phe
2	1.80	98.9	Ala
3	1.84	102	Leu
4	1.91	104	Pro
5	1.29	67.5	Gln
6	1.47	114	Tyr
7	0.99	67.3	Leu
8	0.24	24.2	Lys
9	Trace	—	—
Average yield		80.0	

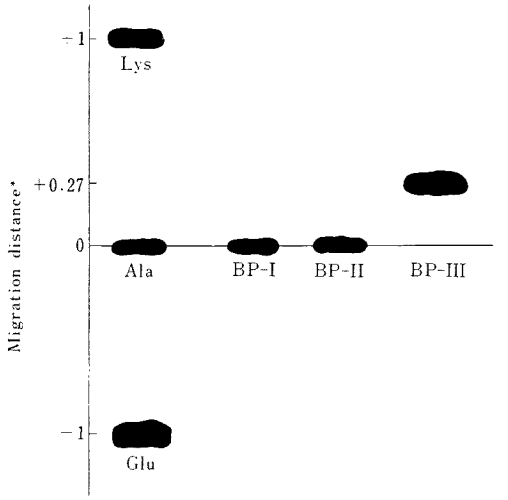

FIG. 5. High-voltage Electrophoresis of BP-I, BP-II and BP-III.

Electrophoresis was carried out at pH 6.5 and 40 V/cm for 45 min.

* Movement relative to alanine (=0), lysine (=+1), glutamic acid (=−1).

Glutamic acid in BP-II and glutamine* in BP-III were further confirmed by their mobility in high voltage paper electrophoresis at pH 6.5 (Fig. 5).

The sequence near the C-terminal region

* Glutamic acid proposed in a preliminary paper[5] should be corrected.

TABLE V. CARBOXYPEPTIDASE DIGESTION OF BITTER PEPTIDES

Peptides	Conditions	Released amino acids (%)
BP-I	CPA for 15 min	Val, Ileu 45, 59 (-Val-Ileu)
BP-II	CPB for 30 min	Phe, Gly, Lys 49, 52, 61 (-Phe-Gly-Lys)
BP-III	CPB for 30 min	Tyr, Leu, Lys 22, 27, 88 (-Tyr-Leu-Lys)

TABLE VI. AMINO ACID SEQUENCES OF BITTER PEPTIDES

BP-I	Gly-Pro-Phe-Pro-Val-Ileu
BP-II	Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys
BP-III	Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys

of the peptides was analyzed by carboxypeptidase digestion. Results were in agreement with the estimation using Edman degradation, as shown in Table V. In the table the amino acid released on digestion with carboxypeptidase is expressed as a percentage of the original peptide present in the digestion mixture.

On the basis of these results, the whole sequence of the bitter peptides is proposed as shown in Table VI.

Tastes of phenylalanine, tyrosine and their derivatives

The existence of phenylalanine, common to the peptides, suggests that this amino acid may be a functional residue in the development of bitter taste. So, 0.1% aqueous solutions of phenylalanine and its derivatives were tasted and compared as to bitterness. Phenylalanine is known to have slightly bitter taste, but its bitterness was strongly increased when its amino and carboxyl groups were blocked by acetyl and ethoxy groups, respectively. Bitterness of phenylalanine and its derivatives was as follows.

TABLE VII. AMINO ACID COMPOSITION OR SEQUENCE OF ISOLATED BITTER PEPTIDES

Origin	Amino acid composition of sequence	Author
Gouda cheese	Phe,*) Pro, aliphatic-amino acids	Raadsveld (1953)[17]
Tryptic hydrolysate of casein	Ala, Leu,*) Phe,*) Asp, Glu, Tyr, Gly, Pro	Carr *et al.* (1956)[16]
Proteolytic hydrolysate of casein	Ala, Leu,*) Phe,*) Glu, Arg,*) Ser, Thr, Tyr, Met,*) Gly, Pro	Ichikawa *et al.* (1959)[18]
Cheese	Val (4), Leu*) (2), Ileu*) (2), Phe*) (1), Glu (2), Arg*) (1), Gly (2), Pro (5)	Gordon and Speck (1965)[19]
Malted bean (Natto)	Ala, Val, Ileu,*) Asp, Glu, Lys, Arg,*) Ser, Tyr, Met,*) Gly, Pro	Maekawa and Tamai (1965)[20]
Peptic hydrolysate of soy bean protein	Gly-Leu,*) Leu*)-Phe,*) Ser-Lys-Gly-Leu,*) Arg*)-Leu,*) Phe*)-(Ileu,*) Leu_2*))-Gln-Gly-Val, Leu*)-Lys, Arg*)-Leu*)-Leu*)	Fujimaki *et al.* (1968)[3]
	Pyrrolidone carboxyl-Gly-Ser-Ala-Ileu*)-Phe*)-Val-Leu*)	Yamashita *et al.* (1968)[4]

*) Amino acids having bitter taste.

$$Phe \approx Ac\text{-}Phe < Phe \cdot OEt < Ac\text{-}Phe \cdot OEt$$

0.1% aqueous solutions of tyrosine and its derivatives were also tasted. Although tyrosine was faintly bitter, or tasteless, tyrosine ethylester and N-acetyl tyrosine ethylester possessed strong bitter tastes. Bitterness of tyrosine and its derivatives was as follows.

$$Tyr \approx Ac\text{-}Tyr < Tyr \cdot OEt < Ac\text{-}Tyr \cdot OEt$$

These results suggest that amino and carboxyl groups, especially the latter, in tyrosine and phenylalanine may contribute to weakening the bitterness of these amino acids. Therefore, the bitterness of these amino acids possibly is intensified when they are introduced in small peptides.

DISCUSSION

The amino acid compositions or the primary structures of various bitter peptides, isolated from enzymatic hydrolysate of protein, are summarized in Table VII. These bitter peptides, BP-I, BP-II and BP-III, were different from other bitter peptides (Table VI). However, it is apparent that all the peptides listed contain amino acids which have bitter tastes in their free states (marked with a star). Furthermore, these peptides are characterized by high contents of an amino acid with a hydrophobic side chain. Diffusible bitter peptides and ninhydrin negative bitter peptides, which were isolated by Fujimaki's group,[3,4] have leucine as the N- or C-terminal. Our peptides contain phenylalanine and proline in common. BP-I and BP-II possess Pro-Phe-Pro sequences. BP-II and BP-III have phenylalanine at the N-terminal and lysine at the C-terminal. High contents of proline and N-terminal lysine in these peptides may be attributable to substrate specificity of trypsin.

Generally, the threshold[21] of bitterness in free amino acids depends on concentrations of more than 0.1%. The peptides isolated here showed stronger bitterness than was

17) W. Raadsveld, *Intern. Dairy Congr., Proc. 13 th (Hague)*, **2**, 676 (1953).

18) K. Ichikawa, T. Yamamoto, M. Nishio and J. Fukumoto, *Nippon Nogeikagaku Kaishi*, **34**, 448 (1960).

19) D. F. Gordon, Jr and M. L. Speck, *Appl. Microbiol.*, **13**, 537 (1965).

20) K. Maekawa and S. Tamai, Abstracts of Paper, 36 th National Meeting of Agricultural Chemical Society of Japan, Tokyo, April, 1965.

21) M. Yoshida, M. Ohara, T. Ninomiya, S. Ikeda and S. Yamaguchi, Abstract of Paper, 36 th National Meeting of Agricultural Chemical Society of Japan, Tokyo, April, 1965.

expected from the taste of the amino acid which made up their compositions. Position of bitter amino acids in peptides may be important for development and/or strengthening of bitter taste.

Fujimaki *et al.*[3] suggested that leucine in the terminal position of bitter peptides may be important for bitter taste. Minamiura *et al.*[22] degradated a bitter peptide stepwise from its N-terminal using neutral amino peptidase of *B. subtilis*. They found that bitter taste disappeared when the tyrosine residue in the peptide was removed. All these results show that the position of amino acid in a peptide is significant for the development of bitter taste.

Bitter tastes of tyrosine and phenylalanine were significantly intensified when the amino and carboxyl groups of these amino acid were, respectively, acetylized and esterified. This shows that the position of bitter amino acid in peptide is important for strengthening the bitter taste. Furthermore, since tyramine and tyrosol are strong bitter substances, amino and carboxyl groups would have quenching effects on the bitterness of tyrosine.

Tokita *et al.*[23] described that their bitter peptide possesses a cyclic structure, such as azlacton or oxazolone, which is functional for bitter taste. Carr *et al.*[16] presumed that bitter peptide has a cyclic portion with a side chain carrying a C-terminal amino acid. Yamashita *et al.*[4] isolated bitter peptide having pyrrolidone carboxyl glycine in the N-terminal. However, the relationship between bitterness and cyclic structure was not clarified in the latter two studies. The bitter peptides in our study do not contain a cyclic structure, as described above.

Acknowledgements. We wish to express our thanks to Dr. K. Hayashi, Assistant Professor of Kyoto University, for his kind guidance in the sequential analyses of peptides and to Mr. T. Higasa, the Research Institute for Food Science, Kyoto University, for his technical assistance in amino acid analyses.

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